ABSTRACT

Catalase-peroxidase (KatG) is an enzyme capable of utilizing both catalase and peroxidase activity to break down hydrogen peroxide. Catalase activity by the enzyme generally dominates, but peroxidatic electron donors have been shown to stimulate catalase activity, likely by rescuing catalase-inactive intermediates resulting from off-pathway electron transfer. To investigate tryptophan 438 as a potential conduit for this misdirected transfer, a variant form of the enzyme (W438F), which contained a phenylalanine in place of the tryptophan, was produced by site-directed mutagenesis, expressed, and purified. Catalase and peroxidase activities were measured via UV-Vis spectrophotometry, and catalase activity was also monitored by oxygen production. We observed a threefold increase in catalase activity by the variant as compared to wild-type KatG. Additionally, W438F displayed a threefold decrease in peroxidase activity. These results are consistent with the possibility that off-pathway electron transfer could occur by this route, as the phenylalanine substitution would obstruct this oxidizable passage and cause a diminished requirement for the peroxidatic rescue event. However, oxygen production data also revealed stimulation of catalase activity by peroxidatic electron donors at pH 5, and further investigation is needed to understand this pathway.

INTRODUCTION

The enzyme catalase-peroxidase (KatG) is found in a wide range of lower eukaryotes and bacteria, including Mycobacterium tuberculosis. KatG from M. tuberculosis (MtKatG) has received the most attention because it is required for the activation of the frontline antitubercular agent isoniazid. An alarming trend in tuberculosis has been the increasing prevalence of multi- and extensively drug-resistant strains (World Health Organization, 2013). Isoniazid is one of the most widely used treatments against tuberculosis, but over 70% of the M. tuberculosis strains resistant to isoniazid are the result of mutations to the KatG gene (Jagielski et al., 2013). In all organisms that carry it, the primary function of KatG is to degrade hydrogen peroxide (H₂O₂). Due to its potential to damage cellular components and create free radicals, H₂O₂ must be removed by all organisms that live in an aerobic environment. Pathogens in particular require a mechanism to decompose H₂O₂ because this compound is a central player in host immune responses. KatGs are found in a number of human and plant pathogens, and they are often associated with other events and virulence factors (Bandyopadhyay & Steinman, 2000; Brunder et al., 1996; Garcia et al., 1999).

Throughout biology there are two principal mechanisms for H₂O₂ decomposition, catalase and peroxidase. In a typical catalase reaction, one unit of H₂O₂ is reduced to H₂O, followed by the oxidation of a second unit of H₂O₂ to produce molecular oxygen (O₂) (Reaction 1). The first step of the peroxidase pathway similarly...
involves the reduction of $\text{H}_2\text{O}_2$, but in the second step an exogenous electron donor is oxidized instead (Reaction 2).

**Reaction 1:**

$$2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2$$

**Reaction 2:**

$$\text{H}_2\text{O}_2 + 2 \text{RH} \rightarrow 2 \text{H}_2\text{O} + 2 \text{R}^*$$

Interestingly, there is almost no overlap between these two pathways. That is, enzymes with robust catalase activity are uniformly poor as peroxidases and vice versa. KatG stands as the lone exception as it is capable of both activities.

After intense investigation, it is clear that KatG carries out its catalase activity by a mechanism distinct from typical monofunctional catalases, but this mechanism and its interplay with the enzyme’s peroxidase mechanism remain elusive (Njuma et al., 2014). However, each mechanism yet proposed predicts mutual antagonism between KatG’s catalatic and peroxidatic mechanisms. That is, peroxidatic electron donors should inhibit KatG catalase activity. Contrary to these predictions, we have previously shown that peroxidatic electron donors can stimulate catalase activity by an order of magnitude (Ndontsa et al., 2012).

KatG is a heme protein, and although its mechanism is not yet understood completely, it is known that a series of highly reactive and oxidizing intermediates is involved, including ferryl iron states (i.e., Fe$^{IV}$=O) as well as heme- and protein-based free radicals (Njuma et al., 2014). KatG has been shown to contain a novel structure where the side chains of a tryptophan (W), a tyrosine (Y), and a methionine (M) are covalently linked to produce a MYW adduct (Yamada et al., 2002). The MYW adduct appears to function as a redox-cycling cofactor in KatG, alternating between its fully covalent (reduced) and free radical (oxidized) states. Due to these highly reactive and oxidizing intermediates, there exists a possibility for off-pathway electron transfer, where one of these compounds incorrectly oxidizes another part of the protein. Having improperly gained an electron, the enzymatic intermediate would become functionally inactive and unable to continue its cycle, resulting in the loss of activity. However, a peroxidatic electron may be able to return this catalase-inactive intermediate to the resting ferric form to once again participate in active turnover. In this manner, the peroxidatic donor would serve to “rescue” the enzyme, resulting in the observed stimulation of catalase activity. Peroxidase activity itself, then, could actually be considered a manifestation of misdirected electron transfer that occurs during catalatic turnover (Ndontsa et al., 2012).

One potential route for misdirected electron transfer would be tryptophan 438 (W438). This residue is located near the surface of the protein, allowing for access to exogenous electron donors (Figure 1). W438 is also in close proximity to arginine 418 (R418), which is known to undergo a pH-dependent conformational shift that could potentially permit increased off-pathway electron transfer.
transfer (Carpena et al., 2006; Zhao et al., 2012). To evaluate W438 as an off-pathway electron transfer route, we used site-directed mutagenesis to replace W438 with phenylalanine (F), producing W438F KatG. By replacing oxidizable tryptophan with a non-oxidizable phenylalanine, this route for electron transfer would be blocked. As a result, fewer inactive intermediates would accumulate and require rescue by peroxidatic electron donors. As such, the W438F KatG variant would be expected to exhibit increased catalase and decreased peroxidase activity as compared to the wild-type enzyme.

**EXPERIMENTAL METHODS**

**Mutagenesis, Expression, and Purification of the W438F Variant**

All materials were purchased as described previously (Ndontsa et al., 2012). Site-directed mutagenesis also followed the procedure outlined in prior reports (Wang & Goodwin, 2013). The primers TTCAAGATCCGGTCCCTGCG & CAGGCTCTGCTTGGGAC, which contained a phenylalanine codon replacing that of the tryptophan, were used to introduce the mutation. Plasmids were screened by restriction digests using BamHI and HindIII. Candidate plasmids were sent to Davis Sequencing for sequence analysis, where it was confirmed that the only substitution occurred at the 438 site. Expression of the W438F variant was carried out using C41 [DE3] cells bearing the pHPEX3 plasmid (Varnado & Goodwin, 2004) as previously described for wild-type MtKatG (Ndontsa et al., 2012). Briefly, Luria-Bertani medium was supplemented with ampicillin (100 μg/mL) and tetracycline (20 μg/mL). Cultures (1 L) were grown to mid-log phase (OD_{600} 0.4-0.6), at which point expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Hemin (8 μM) was also added at the time of induction to ensure that the protein expressed would include its heme prosthetic group.
At four hours post-induction, cells were harvested by centrifugation and cell pellets stored at -80°C until purification. Analyses of the expression revealed that W438F KatG was expressed in a soluble form. Therefore, purification of the protein was carried out by nickel affinity and anion-exchange chromatography as previously described for the wild-type enzyme (Ndontsa et al., 2012).

**Magnetic Circular Dichroism (MCD)**

All spectra were obtained using 15 μM enzyme in 50 mM phosphate, pH 7.0, 50 mM NaCl. Spectra were measured using a Jasco J-810 spectropolarimeter (Tokyo, Japan) using a 1.4 Tesla magnetic cell holder. The ferrous state was obtained by the addition of sodium dithionite.

**Peroxidase and Catalase Activity Assays**

Peroxidase activity was measured by monitoring the production of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical over time at 417 nm (ε_{417} = 34.7 M^{-1} cm^{-1}) (Scott et al., 1993). Initial velocities were determined for a range of H_2O_2 concentrations while enzyme (20 nM) and ABTS (0.1 mM) concentrations were held constant. Apparent kinetic parameters were determined by non-linear least squares fitting of the initial velocities to the Michaelis-Menten equation. All assays were carried out at ambient temperature in 50 mM acetate, pH 5.0 using a UV-1601 spectrophotometer (Shimadzu, Columbia, MD).

Catalase activity was measured by two methods. The first monitored the decrease in H_2O_2 spectrophotometrically at 240 nm (ε_{240} = 39.4 M^{-1} cm^{-1}) (Nelson & Kiesow, 1972). All assays contained 20 nM enzyme and were carried out at room temperature in 100 mM phosphate, pH 7.0. The second monitored O_2 production by Clark-type O_2-sensitive electrode. Sodium dithionite and N_2 were used to calibrate the instrument and initially establish zero O_2 in the reaction chamber. All reactions were carried out at room temperature with either 2 or 5 nM enzyme in either 50 mM acetate, pH 5.0 or 100 mM phosphate buffer, pH 7.0. A 20-second baseline was obtained for each assay mixture prior to the addition of H_2O_2. H_2O_2 was then added and O_2 production monitored.

**RESULTS AND DISCUSSION**

The W438F KatG variant was expressed in a soluble form and purified. Because the protein contains heme, a UV-Vis spectrum for the enzyme-bound heme was taken and used to verify the purity of the enzyme. The spectrum for the heme was virtually identical to that of wild type KatG, and the ratio of heme absorbance to general protein absorbance (A_{408}/A_{280}) was consistent with a highly purified preparation of the enzyme (data not shown). To further evaluate the heme environment and thus the structural integrity of the enzyme, magnetic circular dichroism (MCD) was employed. Spectra for the Fe^{III} and Fe^{II} states of W438F KatG showed that the protein contained heme with its iron in a high-spin state, and these spectra were nearly superimposable on
those of wild-type KatG (Figure 2). All of these results pointed to the robust expression of a properly folded KatG indicating the W438F substitution had not produced a global disruption of the KatG structure.

The variant showed about a threefold increase in catalase activity as compared to the wild-type enzyme (Figure 3). The effect of \( \text{H}_2\text{O}_2 \) concentration on catalase activity showed a catalytic efficiency with respect to \( \text{H}_2\text{O}_2 \) (i.e., \( k_{cat}/K_M \)) of \( 1.6 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \) for the variant versus \( 6.2 \times 10^5 \text{ M}^{-1} \text{s}^{-1} \) for the wild type. In terms of the maximum catalytic output of the enzyme (i.e., \( k_{cat} \)), W438F exhibited \( 1.5 \times 10^4 \text{ s}^{-1} \) versus \( 4.4 \times 10^3 \text{ s}^{-1} \) for the wild-type.

In contrast, peroxidase activity diminished threefold as a result of the phenylalanine substitution (Figure 4). In particular, the wild type displayed a \( k_{cat}/K_M \) with respect to \( \text{H}_2\text{O}_2 \) of \( 2.8 \times 10^4 \text{ M}^{-1} \text{s}^{-1} \), significantly higher than that of W438F, \( 7.9 \times 10^3 \text{ M}^{-1} \text{s}^{-1} \). The difference was even more striking in \( k_{cat} \), with a value of \( 4.123 \text{ s}^{-1} \) obtained for the variant, as opposed to \( 50.72 \text{ s}^{-1} \) for the wild type. These findings are in keeping with the original hypothesis, which predicted increased catalase activity by the variant as a result of decreased off-pathway electron transfer. Similarly, peroxidase activity was predicted to decrease due to a diminished need for the peroxidatic rescue mechanism.

**Figure 5.** Effect of the electron donor ABTS on catalase activity at pH 5.0. Activity was monitored by measuring \( \text{O}_2 \) production for W438F in the presence (▲) and absence (△) of 0.1 mM ABTS. The same was carried out wild type in the presence (●) and absence (○) of 0.1 mM ABTS. All W438F reactions contained 2 nM enzyme, and wild-type KatG reactions contained 5 nM enzyme. All assays were carried out in 50 mM acetate buffer, pH 5.0, at room temperature.

**Figure 6.** Effect of the electron donor ABTS on catalase activity at pH 7.0. Activity was monitored by measuring \( \text{O}_2 \) production for W438F in the presence (▲) and absence (△) of 0.1 mM ABTS. The same was carried out wild type in the presence (●) and absence (○) of 0.1 mM ABTS. All W438F reactions contained 2 nM enzyme, and wild-type KatG reactions contained 5 nM enzyme. All assays were carried out in 100 mM phosphate, pH 7.0, at room temperature.
Catalase activity was also monitored by O\textsubscript{2} production, allowing for measurement of catalase activity even in the presence of a peroxidatic electron donor. The overwhelming absorbance of these compounds where H\textsubscript{2}O\textsubscript{2} consumption is typically measured prohibits the use of the spectrophotometric method for these experiments. Previous data have shown that optimal unassisted catalase activity occurs at pH 7, while maximal peroxidase activity and electron donor stimulated catalase activity occur at approximately pH 5 (Moore et al., 2008). Accordingly, the rate of O\textsubscript{2} production by the enzyme was monitored at these two pH values to determine the effects of pH and an electron donor on the catalase activity of wild-type and W438F KatG.

At pH 5, the variant and wild type alike showed substantial stimulation upon addition of an electron donor (Figure 5). However, there appears to be little difference in enzymatic activity between the wild type and W438F in either situation. Interestingly, the behavior of both the wild type and W438F slightly deviated from standard Michaelis-Menten kinetics, in a similar manner but under different conditions. Both enzymes displayed an initial hyperbolic response in activity, which became linear at higher concentrations of H\textsubscript{2}O\textsubscript{2}. This behavior was displayed by the wild type at pH 5 in the absence of electron donors, but by W438F at pH 5 only when electron donors were present. In contrast, at pH 7 the addition of electron donor inhibited W438F catalase activity (Figure 6). In the absence of an electron donor at pH 7, W438F displayed a greater rate of O\textsubscript{2} production (i.e., catalase activity) than the wild type enzyme. Without the complications of off-pathway transfer, we would expect similar inhibition by electron donor as the result of peroxidase activity competing with catalase activity. However, the pH 5 data show that catalase activity by the variant was enhanced by the electron donor.

The data obtained thus far from the W438F variant have illuminated a novel aspect of the overlap between the catalatic and peroxidatic mechanisms of KatG. This variant exhibits increased catalase activity and decreased peroxidase activity as compared to the wild-type enzyme. If indeed the W438 residue is a route of off-pathway electron transfer, the presence of phenylalanine in place of tryptophan would prevent its oxidation by ferryl iron, the MYW adduct radical, or similarly reactive intermediates. With decreased electron transfer, fewer catalase-inactive intermediates would accrue and there would be a diminished need for peroxidatic rescue events. Concomitantly, as a result of lesser accumulation of inactive intermediates, the enzyme would be able to complete and then repeat its catalatic cycle more easily and efficiently. As such, these data support the original hypothesis.

Nevertheless, further evaluation is needed to determine the extent of the effects of pH and electron donors. In addition to more steady-state kinetic analyses, techniques such as stopped-flow spectrometry and rapid freeze-quench electron paramagnetic resonance should be employed to examine the effects of the W438F substitution. These methods will allow an in-depth examination of which heme, MYW adduct, and protein radical intermediates predominate during and following H\textsubscript{2}O\textsubscript{2} decomposition. The high frequency of oxidizable amino acids in the structure of KatG suggests that the process of off-pathway electron transfer may involve multiple residues. Therefore, other amino acids ought to be evaluated to understand this multipartite pathway. One notable candidate is tyrosine 113 (Y113), which is immediately adjacent to W438 and in close proximity to R418 within the KatG structure. With this in mind, we will continue to focus our efforts on investigating these routes to better understand the dynamic interplay between the catalatic and peroxidatic mechanisms of KatG.

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REFERENCES

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